## C20 RMP3 Additional information

### Recommendation for positive control / QC materials:

Positive control materials for HIV-1 testing are essential to ensure reliability and consistency across assays and constitute an integral component, thus serve as an indicator of assay performance and encompassing the target of interest.

Recommended HIV-1 RNA positive control material (e.g. ATCC VR-3351SD) can be used. Recommended HIV-1 RNA negative control material e.g. human RNA can be used. The negative control from (Exact Diagnostics Negative Control #NEG000) can also be used but it requires extraction. A negative template control (NTC) consisting of nuclease free water is included in each run.

### Part of measurement interval which requires dilution, recommended diluent and maximum lambda

The measurement interval of the RMP to HIV-1 RNA is $10^2$ to $10^4$ copies/µL (CCQM-P199).

Application of the RMP to an EQA HIV-1 scheme covered a wide concentration range of HIV-1 samples, from 42 copies/mL to 37,000 copies/mL (as reported in Falak et al., 2021). The dPCR performance criteria are measured as a maximum lambda below 0.7, which allows for effective separation of positive and negative partitions. This enables both rainfall and droplet dispersion to be measured without being biased by the fact that one population is larger than the other (DOI). For samples > $1 \times 10^3$ copies/µL, it is recommended to dilute 1:5 in nuclease-free water.

### Recommendation for dPCR threshold setting

Setting the dPCR threshold is a critical step for ensuring accurate and reproducible quantification of the RNA target. The threshold setting in dPCR refers to the fluorescence signal value that distinguishes positive droplets (targeted molecules) from negative droplets (no target present). Thresholds setting for the 1D plot can be accomplished by selecting either a single threshold and subsequently clicking on the droplet plot.

Thresholds are set with reference to the positive and negative controls as suggested by ISO 20395 (2019).

PTB recommendation for the threshold settings:

1. Fluorescence signal characteristics:

O The threshold should be set where there is a clear separation between the positive (fluorescent) and negative (non-fluorescent) partitions, with minimal overlap between them.

O In case of poor separation of positive and negative droplets, the threshold should be set just above the signal level indicative for negative droplets to be low

enough to ensure that all based on the appearance of positive and negative controls are counted.

2. Manual vs Automatic threshold setting:

Manual threshold is recommended, especially when the automatic threshold does not properly account for slight shifts in fluorescence signals or background noise.

3. Optimal threshold range:

Typically, the threshold is set at the line where the fluorescence signal sharply rises from the background signal.

## Partition volume and uncertainty

The partition volume (Vp) specified by the RMP (0.854 nL) should be used unless measured directly in-house. Based on CCQM-P199.b, partition volumes of between 0.76 nL and 0.79 nL were reported using direct measurements of the Bio-Rad 1-step RT-dPCR reagents. Based on the lower value minus Bio-Rad value (0.76-0.85), standard uncertainty based on the half range of 0.09 nL/sqrt (3) is 0.052 nL (6.1% relative standard uncertainty).

## Experimental design

The experiments should be performed on four different days using the same materials. Each run should include at least six technical repeats, aside of positive and negative controls. In total, at least 24 technical repeats should be performed for each sample.

## Measurement uncertainty equation(s)

The following factors that contribute to the measurement uncertainties, along with the relevant formulae, were utilised during the course of the P199 study to determine measurement uncertainty: precision (Type A uncertainty) ($u_{r,1}$) and pipetting error / dilution ($u_{r,2}$) and droplet (dPCR partition) volume ($u_{r,3}$). For each factor the relative uncertainty $u_{r,}$ and degrees of freedom ($\nu$) were calculated and the effective degrees of freedom $\nu_{eff}$ was determined using the Welch-Satterthwaite formula (GUM JCGM 1000 Appendix, G.4.1). The combined uncertainty ($u_{r,c}$) and effective expansion factors ($k_{eff}$) were calculated using the following equations.

Eq.1 $$u_{r,c} = \sqrt{u_{r,1}^2 + u_{r,2}^2 + u_{r,3}^2}$$

Eq.2 $$\nu_{eff} = \frac{u_{r,c}^4}{\frac{u_{r,1}^4}{\nu_1} + \frac{u_{r,2}^4}{\nu_2} + \frac{u_{r,3}^4}{\nu_3}}$$

Eq.3 $$k_{eff} = T_{inv}(0.05, \nu_{eff} - 1)$$

The expanded uncertainty ($U$) was calculated using the following equation:

Eq.4 $$U = k_{eff} \times u_{r,c}$$